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A Form of Cytochrome b_5 That Contains an Additional Hydrophobic Sequence of 40 Amino Acid Residues

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ABSTRACT A species of cytochrome b_5 with a monomer molecular weight of 16,700 has been isolated from rabbit-liver microsomes by a procedure that uses detergents and avoids the use of any proteolytic or lipolytic enzymes. This detergent-extracted cytochrome b_5 is larger than the trypsin- or lipase-extracted enzyme, and appears to contain an extremely hydrophobic appendage of 40 amino acids, probably at the N-terminus. The hydrophobic character of the extra amino acid sequence leads to aggregation in the absence of detergents, and may be of considerable importance in the binding of the enzyme to microsomes. It is suggested that the hydrophilic portion of the cytochrome molecule, which bears the heme and is enzymatically functional, is oriented toward the surface of the membrane where it readily reacts with nonmicrosomal proteins, while the hydrophobic "tail" anchors the heme protein tightly to the membrane.

Cytochrome b_5 is a well-characterized component of mammalian and avian liver microsomes. In several species, lipase or trypsin extraction of microsomes allows the isolation of cytochrome b_5 with a molecular weight of about 11,000 (1–4). The amino acid sequences of several of these preparations are quite similar (5). In the case of cytochrome b_5 from calf liver, the details of tertiary structure have been examined by x-ray crystallography (6). All of these investigations have utilized cytochrome b_5 derived from microsomes by some type of hydrolytic cleavage, and it has been suggested that these preparations may be proteolytic fragments of a larger molecule (7, 8). Indeed, higher molecular weight preparations of cytochrome b_5, in the range of 16–19,000, have been reported (9). Ito and Sato (8) described a form of cytochrome b_5 that readily polymerizes, has an apparent monomer molecular weight of 25,000, and is isolated from microsomes with detergents.

Because of these variations in the reported molecular weight of cytochrome b_5, and because of an underlying interest in the native structure of membrane components, we have reinvestigated methods for membrane solubilization that do not involve cleavage of covalent bonds. Through the sequential use of nonionic and anionic detergents at low temperatures, we have purified and partially characterized a form of cytochrome b_5, with a monomer molecular weight of 16,700, from rabbit liver; it differs from lipase- or trypsin-extracted heme protein by the addition of a single hydrophobic peptide, probably at the N-terminus. A partially purified preparation of cytochrome b_5 reductase has also been obtained by this same method.

MATERIALS AND METHODS

All reagents were of the highest commercially available grade and were used as obtained. Cytochrome b_5 was assayed in a Cary 14 spectrophotometer; an extinction coefficient of the oxidized Soret band of 117,000 was assumed. In cruder preparations, where other chromophores interfere and where cytochrome b_5 reductase is present, the cytochrome b_5 content was estimated from the difference between the DPNH-reduced and the oxidized spectra at 423 nm (extinction change = 100,000). When needed, Triton X-100 was added to clarify turbid solutions (final concentration, 1%). Cytochrome b_5 reductase was assayed by the method of Strittmatter and Velick (10), in which ferricyanide acts as an electron acceptor. The tryptophan and tyrosine content of purified cytochrome b_5 was measured by the method of Edelhoch (11). Amino acid analysis was performed by the technique of Spackman, Stein, and Moore (12). Acrylamide disc gel electrophoresis was performed in Tris–glycine buffer (pH 8.9) (13), and in sodium dodecyl sulfate–phosphate buffer (pH 7.5) for the determination of molecular weight (14). Molecular weight was also determined by the high-speed equilibrium method (15) with the aid of a UV photoscanner. These measurements were made in the presence of 0.2% sodium dodecyl sulfate, and, as expected, gave rise to molecular weights that were elevated due to amphiphile bound to the protein. However, since Reynolds and Tanford (16) have shown that a wide variety of proteins bind equal amounts of sodium dodecyl sulfate (on a weight basis), molecular weights can be assigned to unknowns by comparison with molecular weights obtained for standard compounds run under the same conditions. In these experiments, we have employed the lipase-extracted form of apocytochrome b_5 as a standard (molecular weight = 11,100) and have calculated the molecular weight of the detergent-extracted cytochrome b_5 from the relationship:

$$11{,}100 \times (d \log C \text{ detergent-extracted } b_5/dr^2) \div (d \log C \text{ lipase } b_5/dr^2).$$

It should be noted that the assumptions involved in this treatment of ultracentrifugal data are entirely analogous to those underlying the sodium dodecyl sulfate–acrylamide technique for molecular weight determinations.

Lipids were analyzed by thin-layer chromatography on silica gel plates in the solvents chloroform–methanol–water 65:25:4, and the upper phase of acetic acid–toluene–water 5:5:1. Lipid extracts for analysis were prepared by extraction of protein solutions with 20 volumes of ethanol–ether 3:1 at 60°C for 1 hr. After centrifugation, aliquots were concentrated to a small volume and spotted on plates. After chromatography, plates were sprayed with phosphomolybdic acid, rhodamine B, bromthymol blue, anisaldehyde–H_2SO_4, or iodine. Protein determinations were performed by the method of Lowry *et al.* (17). Phosphate was measured by the procedure of Chen *et al.* (18).

Preparation of microsomes

All procedures were performed in the cold room at 2–4°C. A rabbit was killed by a blow on the head and its liver was perfused *in situ* with cold 0.25 M sucrose–0.01 M Tris acetate (pH 8.1)–1 mM EDTA. The liver was excised, cleaned, and weighed, and then homogenized with 9 ml of the buffered sucrose per gram of tissue for 30 sec in a Waring Blendor. The homogenate was centrifuged twice at 18,000 × g for 15 min, the pellet being discarded in each case. The supernatant fluid was centrifuged at 120,000 × g for 1 hr so as to sediment the microsomal fraction. The microsomal pellets were then resuspended in 0.1 M Tris acetate–1 mM EDTA buffer (pH 8.1). Solid NaCl was added, to a final concentration of 1 M, and the suspension was sonicated for 2 min. The microsomal fraction was again sedimented by centrifugation at 120,000 × g for 90 min. The microsomal pellets were again suspended in 0.1 M Tris acetate–1 mM EDTA (pH 8.1), and subjected to two additional sedimentation–suspension cycles in Tris buffer in order to remove residual NaCl. Salt extraction, under these conditions, resulted in the removal of about 30% of the protein initially sedimenting in the microsomal fraction, with only minimal losses of cytochrome b_5 or cytochrome b_5 reductase. The volume of microsomal suspension was made equal to the original wet weight of the tissue.

Solubilization of cytochrome b_5 and cytochrome b_5 reductase

25 ml of glycerol was added to 100 ml of microsomal suspension. DPNH was then added (0.05 mg/ml of suspension), followed by the addition of 10 volumes of acetone at −5°C. The resulting mixture was stirred for 30 min. After centrifugation and removal of the supernatant fluid, the protein precipitate was suspended in 100 ml of 0.1 M Tris acetate–1 mM EDTA buffer (pH 8.1), and stirred for 1 hr. This suspension was centrifuged at 48,000 × g for 20 min, the supernatant fluid was discarded, and the precipitate was again suspended in 100 ml of Tris buffer that contained 1.5% Triton X-100. The Triton extraction was allowed to proceed overnight, after which centrifugation at 48,000 × g for 20 min yielded an optically clear supernatant fluid containing cytochrome b_5 and cytochrome b_5 reductase. The pellet was discarded.

Cytochrome b_5 and cytochrome b_5 reductase were separated on a DEAE-cellulose column in 0.1 M Tris acetate–1 mM EDTA buffer, pH 8.1. The cytochrome b_5 was retained, while cytochrome b_5 reductase was not. The reductase fraction thus obtained was about 10-fold purified over the starting microsomes. After the column was washed with several volumes of 0.1 M Tris acetate–1 mM EDTA buffer (pH 8.1), the cytochrome b_5 was eluted with 0.25 M thiocyanate–0.25% deoxycholate–0.01 M Tris acetate–0.1 mM EDTA, pH 8.1.

Cytochrome b_5 was further purified by gel filtration on Sephadex G-75 in the presence of 0.4% deoxycholate–0.01 M Tris acetate–0.1 mM EDTA buffer, pH 8.1. The red, heme-protein fraction was partially included and eluted behind a front-running, brownish band of excluded material. The cytochrome b_5 thus obtained was freed of detergent by passage through a Sephadex G-25 column equilibrated with 0.02 M Tris acetate–0.2 mM EDTA buffer, pH 8.1.

Lipase-extracted cytochrome b_5 was prepared from rabbit liver microsomes as was described (7).

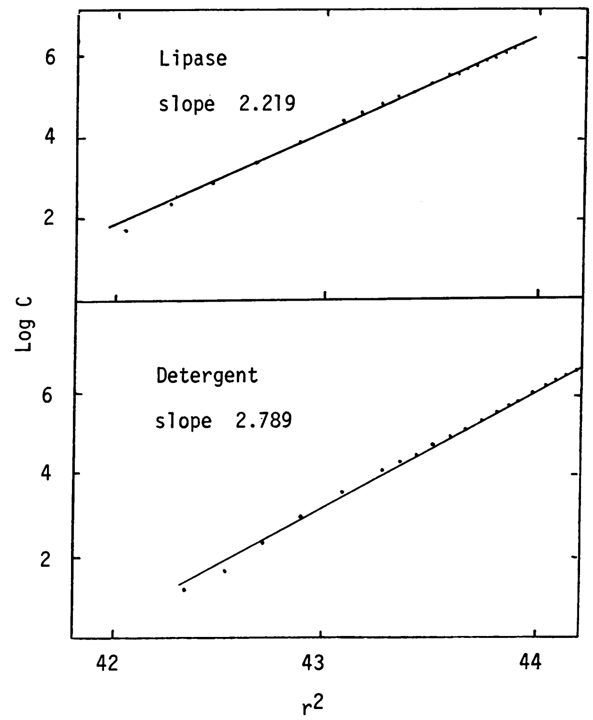

FIG. 1. High-speed equilibrium determinations of the molecular weight of detergent- and lipase-extracted cytochrome b_5. Equilibrium was attained at 18 hr at 44,000 rpm. The solvent is 1.5% sucrose–0.2% sodium dodecyl sulfate–0.02 M PO_4 (pH 7.5). Straight-line plots and slopes were calculated by the least-squares method.

RESULTS

The above procedure permits the isolation of a water-soluble cytochrome b_5 that is homogeneous in the ultracentrifuge (see Fig. 1), gives a single band on sodium dodecyl sulfate–acrylamide disc-gel electrophoresis (Fig. 2*a*), and, in the visible spectral range, is indistinguishable from lipase-extracted cytochrome b_5 in both the reduced and oxidized state (Fig. 3). The overall yield is about 40%. As can also be seen in Fig. 3, the spectrum of the detergent-extracted cytochrome b_5 differs from lipase-extracted cytochrome b_5 only in the near-ultraviolet region. The additional absorption of detergent-extracted cytochrome b_5 is entirely attributable to the additional three tryptophan and two tyrosine residues present in this molecule (Table 1).

Amino acid analysis of this protein shows that it contains 141 residues per heme (Table 1), with a minimum molecular weight (per heme) of 16,100. Lipase-extracted cytochrome b_5 contains only 97 residues per heme. Of the 44 additional amino acids present in detergent-extracted cytochrome b_5, more than 60% are hydrophobic residues. As will be shown, these residues occur largely in a single peptide, which also contains one lysyl and one arginyl residue.

Confirmation of the molecular weight estimated by amino acid analysis was obtained by acrylamide disc gel electro-

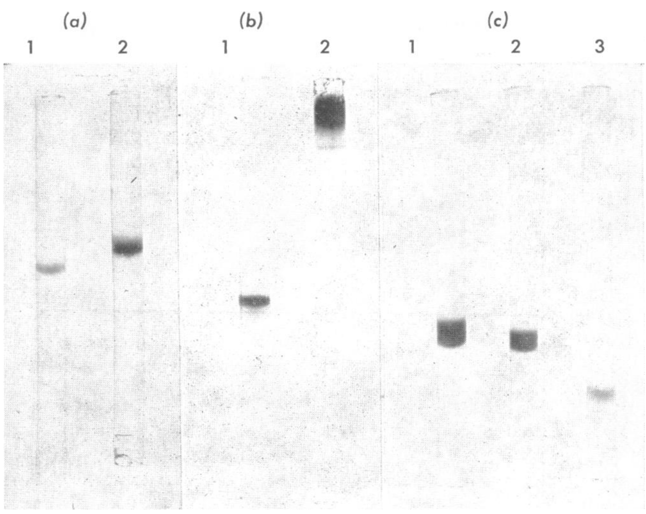

FIG. 2. Acrylamide disc-gel electrophoresis of lipase and detergent cytochrome b_5. (*a*) 15% cross-linked gels were run in sodium dodecyl sulfate–phosphate for 3 hr at 10 mA/tube; (1) lipase-extracted cytochrome b_5, (2) detergent-extracted cytochrome b_5. (*b*) 15% cross-linked gels were run in Tris–glycine for 1 hr at 5 mA/tube; (1) lipase-extracted cytochrome b_5, (2) detergent-extracted cytochrome b_5. (*c*) 15% cross-linked gels were run in sodium dodecyl sulfate–phosphate for 3.5 hr at 8 mA/tube; (1) lipase-extracted cytochrome b_5, (2) heme protein derived from detergent-extracted cytochrome b_5 by tryptic cleavage, (3) hydrophobic peptide derived as in (2). Heme protein and the hydrophobic peptide were separated as described in the text. The hydrophobic peptide stains very poorly, due to its low molecular weight.

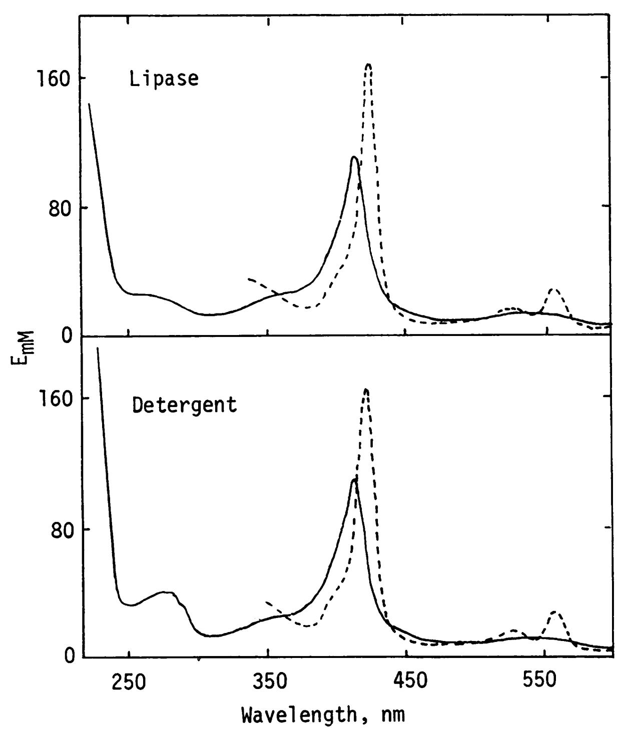

FIG. 3. Spectra of oxidized and reduced detergent- and lipase-extracted cytochrome b_5 in 0.1 M Tris acetate–1 mM EDTA, pH 8.1. Samples were reduced by the addition of purified cytochrome b_5 reductase and DPNH. The *solid line* denotes oxidized spectra; the *dashed line*, the reduced spectra.

phoresis in sodium dodecyl sulfate, with the use of appropriate standards and 15% cross-linked gels. The molecular weight of the apoprotein was found to be 14,500 (Fig. 4) by this technique. Analysis of the data of Fig. 1, as described in *Methods*, similarly indicates a molecular weight for the apoprotein of 14,300. Considering the correction necessary in the latter calculation for the interaction of sodium dodecyl sulfate with the protein, these independent methods show good agreement with the amino acid analysis.

No significant amounts of lipid were found in the detergent-extracted cytochrome b_5 by the chromatographic techniques employed. No lipid-extractable phosphorus could be detected, thus confirming the absence of phospholipids. Lipid analyses were also performed for the detection of residual deoxycholate, and, within the limitations of sensitivity of the method (≥ 5 μM deoxycholate is detectable), none could be found. Amounts of deoxycholate less than this would represent no more than 0.05 equivalent per mole of protein.

Although the molecular weight of monomeric, detergent-extracted cytochrome b_5 is clearly established by these results as 16,700, detergent-extracted cytochrome b_5 readily undergoes polymerization, and is detected in the monomer form only in the presence of sodium dodecyl sulfate. In the absence of detergent, this form of cytochrome b_5 is excluded from Sephadex G-75 gels and has an apparently homogeneous size, on Sepharose 6B gels, of about 120,000. This result indicates polymerization to an octamer. Acrylamide disc gel electrophoresis in Tris–glycine, in the absence of detergent, also suggests that polymerization occurs, since detergent-extracted cytochrome b_5 has considerably less mobility than lipase-extracted cytochrome b_5 (Fig. 2*b*), in spite of the fact that amino acid analysis shows that detergent-extracted cyto-

TABLE 1. *Amino acid composition*

	a Lipase-extracted cyt b_5*	*b* Detergent-extracted cyt b_5†	($b - a$)	Hydrophobic peptide‡
Lys	10	11	1	1
His	7	7	0	0
Arg	3	4	1	1
Asp	10	16	6	4
Thr	7	10	3	3
Ser	7	10	3	3
Glu	14	15	1	1
Pro	3	5	2	2
Gly	6	7	1	1
Ala	5	10	5	4
Cys	0	0	0	0
Val	4	7	3	3
Met	1	3	2	2
Ile	4	8	4	4
Leu	9	15	6	5
Tyr	3	5	2	2
Phe	3	4	1	1
Trp	1	4	3	3
Total	97	141	44	40
Molecular Weight	11,079	16,072	4,993	4,579

* Taken from ref. 7; also verified in the present experiments.
† Average of 4 determinations, rounded to nearest integer.
‡ Average of 3 determinations, rounded to nearest integer.

chrome b_5 may be more negatively charged than lipase-extracted cytochrome b_5. In the presence of 0.4% deoxycholate, detergent-extracted cytochrome b_5 appears to exist as a dimer, with an apparent molecular weight on Sephadex G-75 of about 35,000. Estimates of molecular weight in the presence of urea are somewhat equivocal. Concentrations of urea up to 5 M do not lead to disaggregation, as judged by the continued exclusion from Sephadex G-75 and an apparently large size on Tris–glycine disc gel electrophoresis. In 8 M urea, the mobility of detergent-extracted cytochrome b_5 in Tris–glycine disc gels is increased, though it is still less than lipase-extracted cytochrome b_5. This suggests at least a partial depolymerization.

We have examined the ability of trypsin to convert detergent-extracted cytochrome b_5 to a lower molecular weight, nonpolymerizing form, similar to the heme protein that trypsin releases from microsomes. Tryptic cleavages are generally restricted to lysyl and arginyl residues, and amino acid analysis of detergent-extracted cytochrome b_5 and lipase-extracted cytochrome b_5 indicate the presence of an additional lysyl and an additional arginyl residue in the detergent-extracted protein. It was therefore expected that limited tryptic cleavage of detergent-extracted cytochrome b_5 might produce a heme peptide identical to trypsin-liberated cytochrome b_5, as well as one or two hydrophobic peptides containing the 44 additional amino acids. Furthermore, since trypsin cleaves at the carboxyl side of lysyl and arginyl residues, the disposition of these residues in the peptide fragment(s) may permit assignment of the site of attachment of the peptide(s) to the heme protein. If hydrolytic conditions yield only a single peptide, containing both basic residues, the assignment of the sequence to the N-terminus becomes much less ambiguous. Incubation of a 400-fold molar excess of detergent-extracted cytochrome b_5 with trypsin in 0.02 M Tris acetate–0.2 mM EDTA buffer (pH 8.1), for 18 hr at 25°C, produces a heme peptide that is indistinguishable from lipase-extracted cytochrome b_5 on sodium dodecyl sulfate–acrylamide electrophoresis, as well as one smaller peptide (Fig. 2c). These two peptides have been separated by column chromatography on Bio-gel P-10; in the presence of 0.3% sodium dodecyl sulfate, the larger heme-binding peptide is eluted first, followed by the smaller peptide. On Sephadex G-50, in the presence of 0.1 M Tris acetate–1 mM EDTA (pH 8.1), a water-soluble aggregate of the smaller hydrophobic peptide is excluded and is eluted ahead of the partially included heme peptide. These results clearly show that the observed aggregation of detergent-extracted cytochrome b_5 is a consequence of the properties of the smaller hydrophobic peptide. Amino acid analysis of the small, trypsin-liberated fragment is shown in Table 1. This hydrophobic peptide contains 40 of the 44 additional amino acid residues in detergent-extracted cytochrome b_5, including both the extra lysyl and arginyl residues.

DISCUSSION

The detergent-extracted cytochrome b_5 isolated in these experiments can be compared to the preparation obtained by Ito and Sato (8) by a somewhat similar technique. The minimum molecular weight of 25,000 (based upon heme content) reported by Ito and Sato is higher than that found for our preparation. In view of the overall similarities in methodology between the two preparations, it seems unlikely that our apparently smaller, detergent-extracted cytochrome b_5 results from hydrolytic cleavage. We believe, instead, that the differ-

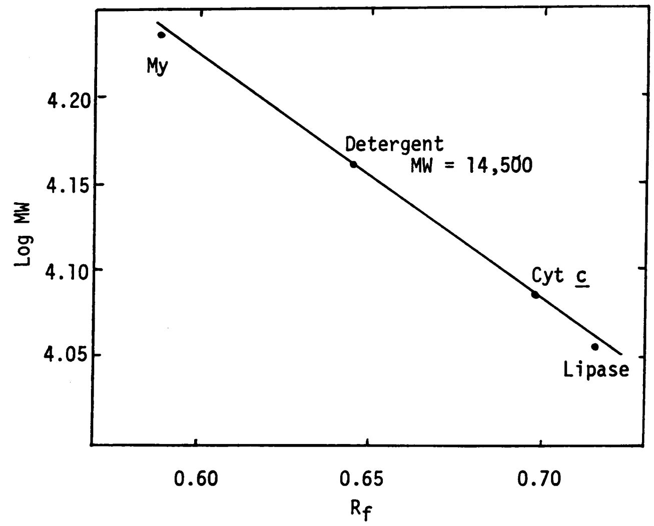

FIG. 4. Molecular weight determination of detergent-extracted cytochrome b_5 by sodium dodecyl sulfate disc-gel electrophoresis. 15% cross-linked gels were run 4 hr at 8 mA/tube. The standards employed were myoglobin, cytochrome c, and lipase-extracted cytochrome b_5. All samples were run in triplicate, and R_f values were averaged.

ences may reflect the use of acetone precipitation by Ito and Sato for the final removal of deoxycholate. In our hands, acetone precipitation of purified detergent-extracted cytochrome b_5 results in some heme loss, and thus an overestimation of the minimum molecular weight based upon heme content could have occurred. Heme loss cannot have occurred in the present preparation, because amino acid analysis does not indicate any additional histidine in detergent-extracted cytochrome b_5 compared to lipase-extracted cytochrome b_5.

Although cytochrome b_5 is tightly bound to microsomes, lipase- and trypsin-extracted cytochrome b_5 show no hydrophobic behavior. The present experiments indicate that microsomal cytochrome b_5, when isolated by procedures that avoid hydrolytic artifacts, has a molecular weight of 16,700 and contains an unusual, extremely hydrophobic, peptide segment of 40 amino acids. It seems likely that this hydrophobic sequence is responsible for the binding of cytochrome b_5 to the microsomal membrane. Although not previously observed, the occurrence of specific hydrophobic segments in otherwise hydrophilic membrane proteins may prove to be a general mechanism for binding these proteins to the membrane. We are presently pursuing this possibility with the crude, detergent-extracted cytochrome b_5 reductase that has previously been purified by procedures involving hydrolytic cleavage.

Data concerning the interactions between cytochrome b_5 and cytochrome b_5 reductase, and the native states of these enzymes in the membrane, are essential for an understanding of electron-transport mechanisms in microsomes. The present studies emphasize that neither cytochrome b_5 nor cytochrome b_5 reductase are covalently bound, since they can be separated from each other by methods that avoid cleavage of covalent bonds. If the cytochrome b_5 isolated in these studies is the native form of the enzyme in the membrane, then questions concerning its state of aggregation *in situ* naturally arise. Since detergents can depolymerize this form of the heme protein, we can ask whether native membrane lipids also have this effect. On the other hand, since there is a large molar excess (10:1) of cytochrome b_5, relative to the reductase, in microsomes, and since the detergent-extracted form of cytochrome b_5 is completely reduced by exogenously added cytochrome b_5 reductase when it is aggregated, it is possible that a polymerized form is native to the membrane.

The incubation of detergent-extracted cytochrome b_5 with trypsin produces a heme peptide, electrophoretically indistinguishable from lipase-liberated cytochrome b_5, as well as a smaller peptide of 40 amino acid residues that contains one lysyl and one arginyl residue. Thus, the conditions chosen for limited tryptic hydrolysis appear to have resulted in a single cleavage, presumably at the carboxyl side of either the lysyl or arginyl residue of this peptide. This leads to the conclusion that the hydrophobic peptide of 40 residues is N-terminal in detergent-extracted cytochrome b_5. X-ray crystallographic studies of calf cytochrome b_5 (6) have shown that the heme-binding crevice and the enzymatically functional portion of the molecule are located at the end opposite to the N-terminus. It is thus tempting to suggest that the hydrophobic amino terminus binds cytochrome b_5 to the membrane, while the hydrophilic, enzymically active portion, is oriented toward the membrane surface and exposed to the environment. This would explain both the tight binding of cytochrome b_5 to the membrane and its observed ability to react rapidly with non-microsomal proteins such as cytochrome *c*.

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